Gene 639 (2018) 18–26

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Association study of apoptosis gene polymorphisms in mitochondrial diabetes: A potential role in the pathogenicity of MD

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ARTICLE INFO

Keywords:
Mitochondrial diabetes
Apoptosis
Polymorphisms
TUNEL
Cytochrome *c*
Protein expression

ABSTRACT

Mitochondrial diabetes (MD) is a heterogeneous disorder characterized by a chronic hyperglycemia and is maternally transmitted. Syndromic MD is a subgroup of MD including diabetic microangiopathy and macroangiopathy, in addition to extrapancreatic disorder. MD is caused by genetic mutations and deletions affecting mitochondrial DNA. This mitochondrial damage initiates apoptosis. In this study, we hypothesized that functional polymorphisms in genes involved in apoptotic pathway could be associated with the development of apoptosis in MD disease and increased its risk. Detection of apoptosis was confirmed on muscle biopsies taken from MD patients using the TUNEL method and the Cytochrome *c* protein expression level. We genotyped then 11 published SNPs from intrinsic and extrinsic apoptotic pathway and assessed the signification of these polymorphisms in 43 MD patients and 100 healthy controls. We found 10 selected polymorphisms (*p53* (rs1042522 and rs17878362), *BCL2* (rs2279115), *BAX* (rs1805419), *BAK1* (rs210132 and rs2227925), *CASP3* (rs1405937), *CASP7* (rs2227310), *CASP8* (rs1045485) and *CASP10* (rs13006529)) with a potential apoptosis effect in MD patients compared to control population. Specifically, SNPs involved in the intrinsic pathway (*p53*, *BCL2*, *BAK1* and *CASP3*) presented the highest risk of apoptosis. Our result proved that apoptosis initiated by mtDNA mutations, can be emphasized by a functional apoptotic polymorphisms associated with high expression of cytochrome *c* protein and more myofibers with apoptosis in syndromic MD subgroup compared with non-syndromic MD subgroup.

1. Introduction

Mitochondrial diabetes (MD), defined as a mitochondrial disease with chronic hyperglycemia due to inappropriate secretion of insulin, insulin resistance, or combined defects(Maassen et al., 2004). In fact, chronic hyperglycemia is a major initiator of diabetic microvascular complications (e.g., retinopathy, neuropathy, and nephropathy) (Sheetz and King, 2002). Syndromic MD is a subgroup of MD associated with hearing impairment, retinal pigment, epithelium damage and even neurological disorders but the nature of the diabetes can be type 1 or type 2 depending on the severity of insulinopenia, (Maassen et al., 2004; Tabebi et al., 2015, 2017). MD is a rare kind of diabetes with a frequency of 1%, characterized by a strong familial clustering of diabetes, discriminated from other type of diabetes based on the presence of maternal transmission in conjunction with a bilateral hearing impairment in most of the carriers (Maassen et al., 2004). Mitochondrial diabetes is provided by genetic analysis. In the large majority of cases, mitochondrial diabetes associates with an A3243G mutation in mitochondrial DNA (mtDNA), although a range of other mutations in mtDNA have also been implicated (Maassen et al., 2005; Mezghani et al., 2010; Mkaouar-Rebai et al., 2009; Tabebi et al., 2017).This mitochondrial damage is a key of morphofunctional characteristic of

Abbreviations: BAK1, BCL2 Antagonist/Killer 1; BAX, BCL2 Associated X, Apoptosis Regulator; BCL2, B-cell lymphoma 2; CASP, Caspases; CI, confidence interval; Cyt *c*, Cytochrome *c*; DNA, DeoxyriboNucleic Acid; Fas, FAS-associated death domain; FasL, FAS ligand; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MD, Mitochondrial diabetes; mtDNA, mitochondrial DNA; OR, Odds ratio; P53, Tumor protein 53 (Tp53); PCR, Polymerase Chain Reaction; SNP, Single-nucleotide polymorphism; TUNEL, Terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin Nick End Labeling

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http://dx.doi.org/10.1016/j.gene.2017.09.063
Received 5 July 2017; Received in revised form 7 September 2017; Accepted 27 September 2017
Available online 04 October 2017
0378-1119/

apoptosis (Elmore, 2007). Indeed, mitochondrial damage is the initial step in caspase-dependent apoptosis, triggered by proteolytic cleavage of BID (Alimonti et al., 2001). During caspase-induced apoptosis, mitochondrial function is completely disrupted, by a loss of the mitochondrial transmembrane potential (ΔΨm). Besides, the outer mitochondrial membrane is disrupted, leading to the release of apoptogenic factors, including cytochrome *c* (Cyt *c*), HtrA2/Omi, endonuclease G (endoG), Smac/Diablo and apoptosis inducing factor (AIF) from the intermembrane space (Martinvalet et al., 2005).

The cells will trigger apoptosis through activation of p53 (Tumor protein 53), CASP9 (Caspase 9) and CASP3 (Caspase 3) proteins (Ko et al., 2004; Lei et al., 1998). Although details of the signaling pathways that trigger apoptosis remain not fully understood, possible mechanisms include also transcriptional activation of the *BCL2* (B-cell lymphoma 2) family members whose BAX (BCL2 Associated X) and BAK1 (BCL2 Antagonist/Killer 1) as a pro-apoptotic proteins and BCL2 as an anti-apoptotic protein (Miyashita and Reed, 1995). These proteins participate in the activation of a sequential signaling that modulates two major apoptotic pathways (Zamzami et al., 1995). One is the intrinsic or mitochondrial pathway, in which the stimuli of p53-BCL2 proteins lead to the activation of CASP9 and the release of cytochrome *c* from the affected mitochondria (Green and Kroemer, 2004). The other, known as the extrinsic or cytoplasmic pathway, which involves a group of proteins such as the membrane-bound FAS ligand (FASL), the FAS-associated death domain (FAS), Caspase 8 (CASP8), and Caspase 10 (CASP10) (Ashkenazi and Dixit, 1998; Lowe et al., 2004). Activation of these two pathways initiates a common downstream proteolytic cascade that involves CASP3 (Caspase 3) and CASP7 (Caspase 7) (Zamzami et al., 1995) which are considered as the execution phase of apoptosis. Remembering that apoptosis is a biological process that regulates physiological cell death and plays an important role in the pathogenesis and the emphasis of a variety of human diseases (Thompson, 1995), we hypothesized that mitochondrial damage caused by mtDNA mutations, initiate apoptosis and the association of functional SNPs of apoptotic genes increase its risk, in mitochondrial diabetes.

For this aim, at first we used TUNEL (terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin Nick End Labeling) assay in patients with mitochondrial diabetes to assess apoptosis implication, evaluate Cyt *c* protein expression level and then we selected and genotyped 11 potentially functional polymorphisms in 9 genes involved in the intrinsic and extrinsic apoptosis pathways (*p53*, *BCL2*, *BAX*, *BAK1 FASL*, *CASP8*, and *CASP10*) as well as effective *CASP*s genes (*CASP3* and *CASP7*). We compared then their allelic and genotypic frequencies with healthy controls to identify their eventual association with mitochondrial diabetes phenotype.

2. Materials and methods

2.1. Study population

Our study focused on 43 Tunisians MD patients (12 men and 31 women). The mean ages were 35.4 ± 5.2 years at disease onset. The clinical examination was carried out in the Department of Endocrinology of University Hospital Hedi Chaker Sfax-Tunisia, and the MD diagnosis was confirmed by genetic study (mitochondrial mutations identification). Among our MD patients, on the one hand, 35 (81.4%) had a non-insulin dependent diabetes and 8 (18.6%) had an insulin dependent diabetes (Table 1). On the other hand, 23 MD patients (53.49%) presented syndromic diabetes. In addition, 100 Tunisian healthy individuals from the same ethnocultural group were tested as controls. These controls have no personal or family history of diabetes or any other disorder. The mean age at analysis was 35.8 ± 2.3 years.

All individuals (patients and controls) provided informed consent as required by the ethics committee of the University Hospital Hedi Chaker, Sfax, Tunisia.

Table 1
Characteristics of Tunisian MD patients.

	Number of patients (%)	Average age of diabetes onset	Sex ratio (F/M)
Non syndromic	**20 (46.51)**	**30.89 ± 4.185**	**17/3**
NIDD	17 (39.54)		15/2
IDD	3 (6.98)		2/1
Syndromic	**23 (53.49)**	**39.9 ± 6.255**	**14/8**
NIDD	18 (41.86)		11/7
IDD	5 (11.62)		3/2

NIDD, non-insulin dependent diabetes; **IDD**, insulin dependent diabetes; **F**, Female; **M**, Male.
Bold values indicate the total result.

2.2. DNA extraction

Total DNA was extracted from peripheral blood leucocytes using phenol-chloroform standard procedures (Lewin and Stewart-Haynes, 1992). The quantity and quality of extracted DNA was sufficient to perform the genotyping of different SNPs.

2.3. SNP selection

We used the National Center for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov), and literature search (Hu et al., 2008) to identify potentially functional variants in genes involved in both intrinsic and extrinsic apoptotic pathways.

2.4. Genotyping

The genotyping methods used to distinguish the 11 selected polymorphisms in 9 apoptosis-related genes are presented in Table 2. The fragments of interest packaging the nucleotide varying studied (SNP) were amplified by PCR (Polymerase Chain Reaction) and were genotyped by PCR, RFLP-PCR (Restriction Fragment Length Polymorphism-PCR) method, direct sequencing and PIRA-PCR (Primer-Introduced Restriction Analysis- Polymerase Chain Reaction) assay (Ke et al., 2001).

For all selected polymorphisms, PCR products containing each target genotype were purified and the sequences were confirmed by direct sequencing.

2.5. TUNEL assay

To detect apoptosis in muscle tissue, we used the TUNEL assay (for terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin Nick End Labeling) with an *in situ* Cell Death Detection kit, Fluorescein (Roche) according to the manufacturer's recommendations. TUNEL assay was conducted to highlight the degradation *in situ* DNA. On sections of muscle biopsies, positive controls were made by incubating some sections with 0.5 mg/ml DNAse I (Promega) at room temperature for 10 min before TUNEL staining. DNAse I treated sections which were incubated with fluorescein-labeled nucleotide mixture, without the addition of terminal deoxynucleotidyl transferase, were used as negative controls. The slides were washed with phosphate buffer saline (PBS) and stained with a 4, 6-diamidino-2-phenylindole (DAPI: 100 µg/l) solution for 20 min at room temperature. Then, were analyzed using a fluorescence Microscope (Axioskop Z plus Zeiss). All the experiments were conducted three times with appropriate controls.

2.6. Western-blot analysis of Cytc c expression in muscle samples

We investigated the Cytochrome *c* protein expression level in three muscle biopsies from a syndromic MD patient, a non-syndromic MD patient and a healthy individual.